Resolution of clonal subgroups among *Neisseria gonorrhoeae* IB-2 and IB-6 serovars by pulsed-field gel electrophoresis

C L Poh, G K Loh, J W Tapsall

Abstract

***Objective*—Analysis of macrorestriction patterns by PFGE to resolve the relatedness of clonal subgroups amongst *N gonorrhoeae* IB-2 and IB-6 serovar strains.**

***Materials and methods*—Nineteen IB-2 and eight IB-6 serovar strains that differed in either auxotype or penicillin sensitivity were isolated over a two and a half-year period from patients attending several STD clinics in Sydney. During this period, a major clone, Wt/IB-2 (FS), established on epidemiological grounds, was circulating amongst homosexual males. The genetic relation of this major clone to the other strains present in the community was determined by pulsed-field gel electrophoretic (PFGE) analysis of DNA restriction fragments. Genomic DNA from the 27 isolates were prepared, digested with *Spe*I and *Bgl*II and the restriction patterns were analysed by contour-clamped homogeneous electric field electrophoresis (CHEF) in a CHEF DRIII equipment.**

***Results*—Phenotypic characterisation of the 27 isolates by the combined use of auxotype, serological characterisation and penicillin sensitivity indicated the presence of subgroups within each of the two serovars. In the present study, PFGE analysis of *SPe*I and *Bgl*II-generated genomic DNA restriction patterns from six of the ten Wt/IB-2 (FS) correlated well with phenotypic characterisation of this major clone. Four of the ten Wt/IB-2 (FS) were found to be clonally-derived variants of this major clone as minor genome variations (less than 3 DNA fragments) were observed. Distinct clones were represented by three Wt/IB-2 (LS) isolates as the DNA fingerprints generated from these were unrelated to the major clone. Analysis of PFGE patterns of 6 Pro/IB-2 isolates showed that one was genotypically identical to the major clone, two were clonal variants and three had significantly different patterns to indicate that they were genotypically unrelated. Wt/IB-6 isolates had heterogenous PFGE patterns that were clearly unrelated to the Wt/IB-2 serovar strains. Within the IB-6 serovar, there were three isolates with the Wt/IB-6 (FS) phenotype that could be considered as clonal variants whilst the rest were genotypically distinct.**

***Conclusions*—PFGE analysis of macrorestriction patterns generated from *Spe*I- and *Bgl*II-cleavage of genomic DNA has enabled the establishment of clonal origins of strains present in the Sydney community during the period of study. The delineation of strains belonging to major A/S groups by PFGE analysis presents a clearer epidemiological picture than phenotypic characterisation alone.**

(*Genitourin Med* 1995;71:145–149)

Keywords: Neisseria gonorrhoeae; clones; electrophoresis

Department of Microbiology, Faculty of Medicine, National University of Singapore, Kent Ridge, Singapore 0511
C L Poh
G K Loh

Department of Microbiology, Prince of Wales Hospital, Randwick, Sydney, Australia 2031
J W Tapsall

Correspondence to:
Dr C L Poh.

Accepted for publication
19 December 1994

Introduction

Gonorrhea is a major sexually transmitted disease and is prevalent in both developed and developing countries. Precise characterisation of *N gonorrhoeae* can provide valuable information concerning gonococcal strain populations in any community, the temporal changes as well as the emergence and spread of antibiotic resistant strains.[1] In the absence of a vaccine, data such as these will be useful for devising effective control measures for gonococcal infections.

Currently, the most widely employed method for differentiation of *N gonorrhoeae* strains is one based on auxotyping and serological (A/S) characterisation[2] with serotyping using monoclonal antibodies to protein I providing far better discrimination than auxotyping.[3 4] However, characterisation of strains by serotyping can encounter problems such as non-typability of strains, availability, batch to batch variation of monoclonal antibody reagents and the reproducibility of coagglutination reactions, especially when using monoclonal antibodies such as 2D6, 2G2 and 6D9.[5]

We have recently developed pulsed-field gel electrophoresis (PFGE) for subtyping *N gonorrhoeae* strains expressing identical auxotype/serovar characteristics and this technique appears promising for epidemiological investigations.[6] In Sydney, Australia there has been an outbreak of gonorrhoea in homosexual men with isolates of the IB-2 serovar and of the non-requiring (Wt) auxotype. An additional phenotypic marker, full sensitivity to penicillin (FS), unusual in Sydney isolates, was also used to characterise these strains in an investigation of this outbreak.[7] Other isolates of the IB-2 serovar which differed in auxotype and/or penicillin sensitivity were also isolated over the same period.

Additionally, strains of the IB-6 serovar, also Wt and FS were isolated. The serovars IB-6 and IB-2 are differentiated solely on different reactions with the 2D6 monoclonal antibody.

This study assessed the ability of the PFGE method to discriminate the phenotype Wt/IB-2 (FS) originally identified on epidemiological grounds, its capacity to differentiate subtypes within the IB-2 serovar and to distinguish the phenotype Wt/IB-2/FS from Wt/IB-6/FS.

Materials and methods

Bacterial strains and culture conditions

Twenty-seven clinical isolates of *N gonorrhoeae* with their respective auxotype/serovar characteristics and penicillin susceptibility phenotypes were listed in tables 1 and 2. The isolates comprised ten selected Wt/IB-2 (FS) strains identified as being epidemiologically linked and nine other strains of the IB-2 serovar (table 1) and 8 of the IB-6 serovar (table 2) with a different penicillin sensitivity or auxotype or both. Strains were grown on modified Thayer-Martin agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, USA) and incubated at 37°C in the presence of 5% CO_2 for 20 h.

Serological characterisation

Strains were serotyped using monoclonals manufactured by Syva (Palo Alto, Calif., USA) according to the nomenclature of Knapp *et al*[8] with six MAbs specific for protein IA (PrIA) and six specific for protein IB (Pr IB).

Antimicrobial susceptibility

The MIC of penicillin for the isolates were determined by the agar dilution method.[9] Isolates were classified as fully sensitive (FS) when the MIC was less than 0·03 mg/l, less sensitive (LS) when the MIC ranged from 0·06 to 0·5 mg/l and resistant (R) when the MIC was found to be greater than 1 mg/l.

Genomic DNA preparation and digestion

Genomic DNA was prepared as described previously.[6] Plugs containing embedded DNA were digested overnight with 20 U of *Spe*I or *Bgl*II in buffers as recommended by the manufacturer (New England Biolabs, Beverly, Massachusetts, USA).

Pulsed-field gel electrophoresis

Plugs containing digested DNA were loaded into a 1% agarose gel and electrophoresed in a contour-clamped homogeneous electric field (CHEF DRIII) apparatus with a hexagonal electrode array (BioRad, Richmond, California, USA) at 14°C. Pulse time was ramped from 1 to 15 s for 8 h and then 15 to 25 s for 16 h following *Spe*I digestion and 1 s to 15 s for 22 h following *Bgl*II digestion. Gels were stained with ethidium bromide and photographed under UV transillumination. *Rhodobacter sphaeroides* 2.4.1. genomic DNA digested by *Ase*I was used as the molecular weight standard.[10]

Results

Auxotype/serovar and penicillin susceptibility patterns

Serological characterisation of the 27 isolates established two homogeneous groups. Nineteen of the isolates were found to belong to the IB-2 serovar and eight were of IB-6. The 19 IB-2 serovar strains were classified into 2 auxotypes (wild-type and proline-requiring) and exhibited 3 different patterns of penicillin susceptibility. Twelve of the 19 strains were fully sensitive to penicillin, 5 were less sensitive and 2 were resistant (table 1). Similarly, two auxotypes were evident amongst 8 IB-6 serovar strains and 4 were fully sensitive to penicillin, 3 less sensitive and one was resistant (table 2).

Genome fingerprints of IB-2 and IB-6 serovars

When *Spe*I-digested preparations of genomic DNA from the 19 IB-2 serovar strains were examined, 12-17 fragments ranging from 2 to 410 kb were observed. *Bgl*II digestion of the same isolates yielded 17–24 fragments of between 2 to 260 kb. Five different *Spe*I patterns were found among the 19 IB-2 strains (table 1) whilst digestion with *Bgl*II produced eight different patterns (table 2). Strains with *Spe*I type 3, 4 and 5 patterns were found to differ from strains with type 1 pattern by up to nine fragments. Two of the four strains with identical *Spe*I restriction patterns (fig 1, lanes C & E) could be further distinguished by *Bgl*II digestion (fig 2, lanes C & E). The group of

Table 1 Phenotypic and genotypic characteristics of N. gonorrhoeae IB-2 serovar strains

Strain No.	Auxotype	Penicillin sensitivity	PFGE pattern	
			SpeI	*Bg/II*
92/049	WT	FS	1	1
92/133	WT	FS	1	2
92/213	WT	FS	1	1
92/215	WT	FS	1	3
92/336	WT	FS	1	2
92/038	WT	FS	1	2
92/239	WT	FS	1	1
92/386	WT	FS	1	1
92/413	WT	FS	1	1
92/320	WT	FS	1	1
91/238	WT	LS	2	4
92/290	WT	LS	3	5
92/411	WT	LS	3	6
92/142	PRO	LS	3	7
92/279	PRO	R	4	8
92/221	PRO	R	5	8
92/053	PRO	LS	1	1
91/302	PRO	FS	1	2
92/421	PRO	FS	1	2

Table 2 Phenotypic and genotypic characteristics of N. gonorrhoeae IB-6 serovar strains

Strain No.	Auxotype	Penicillin sensitivity	PFGE pattern	
			SpeI	*Bg/II*
90/125	WT	LS	6	9
91/347	WT	LS	7	10
92/208	WT	FS	8	11
91/227	WT	FS	9	12
91/236	WT	FS	9	13
91/384	WT	FS	10	12
91/275	WT	R	11	14
91/378	PRO	LS	12	15

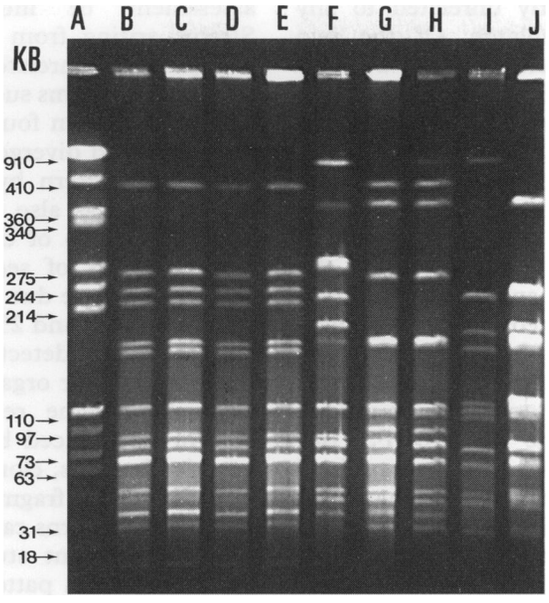

Figure 1 SpeI genome macrorestriction fragments generated from N. gonorrhoeae Wt/IB-2 serovar strains (lane numbers B-J correspond to strains 049, 133, 213, 215, 238, 290, 142, 279 and 221). Lane A: mol. wt marker (R. sphaeroides 2.4.1. digested with AseI).

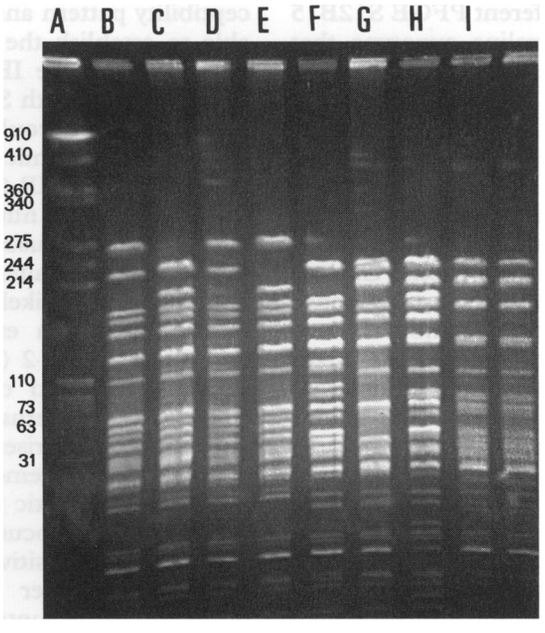

Figure 2 Bg/II genome macrorestriction fragments generated from N. gonorrhoeae Wt/IB-2 serovar strains (lane numbers B-J correspond of strains 049, 133, 213, 215, 238, 290, 142, 279 and 221). Lane A: mol. wt marker (R. sphaeroides 2.4.1. digested with AseI).

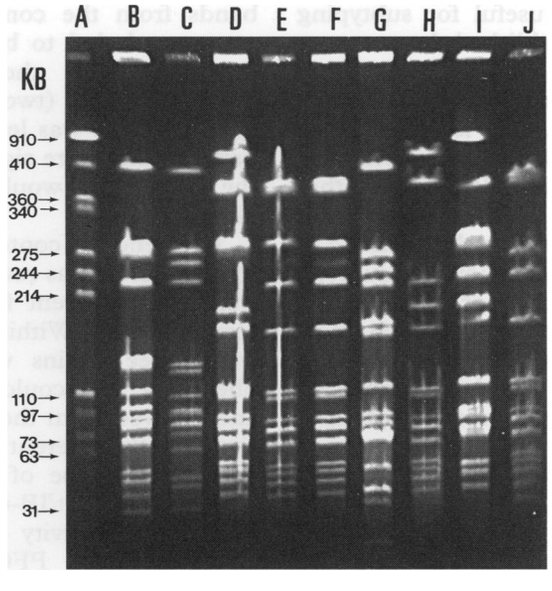

Figure 3 SpeI genome macrorestriction fragments generated from N. gonorrhoeae Wt/IB-6 serovar strains (lane numbers B-J correspond to strains 125, 347, 208, 227, 236, 336, 384, 275 and 378). Lane A: mol. wt marker (R. sphaeroides 2.4.1. digested with AseI).

19 IB-2 serovar strains were found with *Spe*I type 1 pattern. Digestion with *Bgl*II further differentiated the 19 IB-2 strains into seven with type 1, five with type 2, one each with type 3, 4, 5, 6 and 7, and two with type 8 restriction patterns (table 1). The group of five strains with *Spe*I type 1 *Bgl*II type 2 pattern (S1B2) (for example, strain 133; fig 2, lane C) differed from the group consisting of seven strains with *Spe*I type 1 *Bgl*II type 1 pattern (S1B1) (for example, strain 049, fig 2, lane B) in lacking a large DNA fragment at around 259 kb, and the presence of an additional DNA fragment of 195 kb (fig 2, compare lanes B and C). Strain 215 which was assigned with *Spe*I type 1 *Bgl*II type 3 pattern (S1B3) was found to differ from the group of seven strains with S1B1 pattern by the absence of a single DNA fragment of 229 kb (fig 2, lane E) and it also presented an additional fragment of 195 kb which was similarly detected in the group with S1B2 pattern. Strains with *Bgl*II type 4, 5, 6, 7 and 8 patterns showed significant DNA fragment differences when compared with strains showing type 1, 2 or 3 patterns. There were only two to three band differences amongst strains with either *Bgl*II type 1, 2 or 3 patterns but up to 7 band differences were observed between this cluster of strains and strains displaying either type 4, 5, 6, 7 or 8 *Bgl*II restriction pattern.

Digestion of genomic DNAs of the eight IB-6 strains by either *Spe*I or *Bgl*II yielded seven different patterns. The eight IB-6 strains had *Spe*I digestion patterns which were clearly different from the 19 IB-2 serovar strains (fig 3). *Bgl*II digestion again allowed further differentiation of the pair (strains 227 and 236) with identical *Spe*I type 9 pattern (fig 3, E & F) into two closely related *Bgl*II patterns which differed by 3 band differences (data not shown).

Correlation of PFGE data with auxotype and penicillin sensitivity

In the 10 Wt/IB-2 (FS) isolates examined, three different PFGE patterns were found. Six of the ten isolates shared the same S1B1 pattern, three with S1B2 and one had a S1B3 pattern. Three isolates with the non-requiring auxotype (Wt) were less sensitive to penicillin and the PFGE pattern of each isolate was distinct and different from the 10 Wt/IB-2 (FS) isolates. The group of 6 Pro/IB-2 (LS) isolates exhibited three different patterns of penicillin sensitivity. A single Pro/IB-2 (LS) isolate with its S1B1 profile was identical in genome macrorestriction pattern to 6 Wt/IB-2 (FS) isolates (table 1). Two Pro/IB-2 (FS) isolates with S1B2 profiles were found to be identical to three of the Wt/IB-2 (FS) strains (table 1). The remaining three Pro/IB-2 isolates, two of which were resistant to penicillin (92/279 and 92/221) (fig 2, lanes I and J) and one had the LS penicillin phenotype (92/142) (fig 2, lane H), had three different PFGE patterns which were clearly unrelated to any of the Wt/IB-2 (FS) strains (fig 2, lanes B, C and E) or Wt/IB-2 (LS) strains (fig 2, lanes F and G).

The 8 IB-6 isolates had heterogeneous

patterns that were clearly unrelated to any of the IB-2 serovar isolates. Of the two Wt/IB-6 (LS) isolates, one (90/125) was isolated in 1990 and the other (91/347) in 1991. Both were characterised by two different PFGE patterns (fig 3, lanes B and C). Phenotypically, the major group of IB-6 isolates was comprised of four that exhibited the non-requiring auxotype (Wt) and were fully sensitive to penicillin. However, with the exception of isolates 227 and 236, PFGE patterns of the group indicated non-homogeneity in genomic composition (fig 3, lanes D to G). Isolate 92/208 (fig 3, lane D) which was Wt/FS had a PFGE pattern that was unique and different from all the Wt/IB-6 (FS) isolates. One of the Wt/IB-6 isolates examined was resistant to penicillin and examination of the PFGE pattern of this isolate (91/275) by *Spe*I and *Bgl*II digestion clearly established that this strain was very different from the rest of the Wt/IB-6 (FS) isolates. Another isolate that had a PFGE pattern that was unique to itself and clearly different from all the Wt/IB-6 isolates was isolate 91/378 (fig 3, lane J). Besides having a very different PFGE S12B15 profile, it also had a proline auxotype that differentiated this isolate from the rest of the IB-6 serovars.

Discussion

Precise characterisation of *N gonorrhoeae* strains is essential for monitoring and controlling spread of gonococcal infections. Current typing methods adopted in most reference laboratories are based on a combination of phenotypic characteristics such as the auxotypes and serotypes of strains. In strains that carry plasmids, differentiation of strains belonging to the same auxotype-serovar (A/S) groups could be achieved. However, plasmid profiling is of limited value when a common plasmid or a common combination of plasmids are present.[11] In some instances, differentiation of strains present within a common A/S group could be enhanced by including an additional phenotypic trait. As was shown recently by Rowbottom *et al*,[7] an additional phenotypic marker such as sensitivity to penicillin was found to be useful for subtyping Wt/IB-2 isolates and established the presence of a common epidemic clone, Wt/IB-2 that was fully sensitive (FS) to penicillin in Sydney over a period of 2½ years.[7] The isolation of the Wt/IB-2 serovar strains that were less sensitive (LS) to penicillin and Pro/IB-2 strains that were either FS, LS or R during the same period indicated that there is a shortcoming in the use of the A/S scheme alone to subtype strains. However, sensitivity or resistance to penicillin is a phenotypic trait and such alterations in antimicrobial susceptibility without a genetic basis would not be effective in defining clonal subgroups present within a population.

PFGE of genomic DNA of microorganisms following cleavage with restriction enzymes yields DNA fingerprints of every strain. The genomic fingerprints readily provide an assessment of inter-strain relationships. Strains arising from different clonal origins will produce unrelated genome fingerprints. In microorganisms such as *Pseudomonas aeruginosa*, it has been found that clonally derived variants could diverge from a major common restriction pattern by up to six DNA fragments.[12] It was also established in the same study that 56% of changes in antimicrobial susceptibilities of sequential isolates of the same strain were due to variations in chromosome structure and 25% were occurring in the absence of any detectable change in genomic fingerprints. For organisms that can undergo frequent genome rearrangements either *in vivo* or be induced by externally introduced genetic elements, clonal variants that differed by a few DNA fragments in genome macrorestriction patterns can be expected.

In the present study, PFGE analysis of macrorestriction patterns resulting from *Spe*I and *Bgl*II digestion was found to correlate with phenotypic characterisation of the predominant Wt/IB-2 (FS) clone by the A/S classification scheme and antimicrobial susceptibility pattern analysis. In addition, it was able to establish the presence of clonal subgroups within the IB-2 serovar. Thus, three clonal variants with S1B2 and one with S1B3 PFGE pattern could have arisen through some minor genomic mutations or re-arrangements of the Wt/IB-2 (FS) major clone without alteration of nutritional genetic markers and antibiotic susceptibility patterns. The mutational sites altered in these clonal variants would most likely be the recognition sites of the restriction endonucleases. However, two other Pro/IB-2 (FS) clonal variants with similar S1B2 and one Pro/IB-2 (LS) with S1B1 PFGE patterns were found. These variants may have arisen through mutations or genome rearrangements affecting only the proline biosynthetic pathway genes or the β-lactamase gene locus. Alternatively, mutants that were less sensitive to penicillin could also arise from other mechanisms such as decreased drug uptake, alteration of outer membrane permeability and modified gyrase activity.[13] The three Wt/IB-2 (LS) strains had PFGE patterns differing by more than six bands from the common major clone and were concluded to be unrelated in genomic composition to the major clone. Three Pro/IB-2 strains (two were resistant to penicillin and one was less sensitive) with PFGE patterns that were significantly different from the major clone would have been of different clonal origins.

The genomic contents of strains belonging to IB-6 serovar as revealed by PFGE analysis were very different from the group of IB-2 serovar strains. Within the IB-6 serovar, there were three strains with the Wt/IB-6 (FS) phenotype that could be considered as variants derived from the same clone, one had a significantly different PFGE pattern and was considered to be of different clonal origin. Three other Wt/IB-6 strains with differing penicillin sensitivity and one Pro/IB-6 (LS) strain also had PFGE patterns that were

unique and unrelated to the major group of clonal variants.

In conclusion, PFGE analysis of macrorestriction patterns generated from cleavage of genomic DNAs of *N gonorrhoeae* strains with *Spe*I and *Bgl*II correlated well with grouping of strains into either IB-2 or IB-6 serovars which was based on reaction difference observed with the 2D6 monoclonal antibody. The establishment of clonal variants and the identification of distinct clones within each of the two serovars by PFGE analysis would facilitate better monitoring of gonococcal transmission in a community. Additional prospective investigations of isolates derived from cohorts and serial isolates from the same patient will provide further insight into genome variability and the changes in drug resistance observed.

This work was supported by NUS research grant RP 910459 to C L Poh. The authors gratefully acknowledge the supply of monoclonal antibody reagents by Dr J R L Forsyth of the University of Melbourne.

1 Sarafian SK, Knapp JS. Molecular epidemiology of gonorrhoea. *Clin Microbiol Rev* 1989;2(s):49–55.
2 Kohl PK, Ison CA, Danielsson D, *et al.* Current status of serotyping of *Neisseria gonorrhoeae*. *Eur J Epidemiol* 1990;6:91–5.
3 Knapp JS, Sandstrom EG, Holmes KK. Overview of epidemiological and clinical applications of auxotype /serovar classification of *Neisseria gonorrhoeae*. In: Schoolnik GK, Brooks GF, Falkow S, Frasch CE, Knapp JS, McCutchan JA, Morse SA, eds. *The Pathogenic Neisseria*, 1985, 6–12 Washington, D.C., Am Soc Microbiol Press.
4 Poh CL, Ocampo JC, Sng EH, Bygdeman SM. Characterisation of PPNG and non-PPNG *Neisseria gonorrhoeae* isolates from Singapore. *Genitourin Med* 1991;67;389–93.
5 Gill MJ. Serotyping *Neisseria gonorrhoeae*: a report of the fourth international workshop. *Genitourin Med* 1991; 67:53–7.
6 Poh CL, Lau QC. Subtyping of *Neisseria gonorrhoeae* auxotype-serovar groups by pulsed-field gel electrophoresis. *J Med Microbiol* 1993;38:366–70.
7 Rowbottom JH, Tapsall JW, Plummer DC, *et al.* An outbreak of a penicillin-sensitive strain of gonorrhoea in Sydney men. *Genitourin Med* 1994;70:196–9.
8 Knapp JS, Tam MR, Nowinski RC, *et al.* Serological classification of *Neisseria gonorrhoeae* with use of monoclonal antibodies to gonococcal outer membrane protein I. *J Infect Dis* 1984;150:44–8.
9 Tapsall JW, Phillips EA, Cossins YM, *et al.* Penicillin sensitivity of gonococci isolated in Australia: development of the Australian Gonococcal Surveillance Programme. *Br J Venereal Dis* 1984;60:226–30.
10 Suwanto A, Kaplan S. Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1. genome: genome size, fragment identification, and gene localization. *J Bacteriol* 1989;17:5840–9.
11 Dasi MA, Nogueira JM, Camarena JJ, *et al.* Genomic fingerprinting of penicillinase-producing strains of *Neisseria gonorrhoeae* in Valencia, Spain. *Genitourin Med* 1992;68: 170–3.
12 Struelens MJ, Schwam V, Deplano A, Baran D. Genome macrorestriction analysis of diversity and variability of *Pseudomonas aeruginosa* strains infecting cystic fibrosis patients. *J Clin Microbiol* 1993;31:2320–6.
13 Bryan LE. Two forms of antimicrobial resistance: bacterial persistence and positive function resistance. *J Antimicrob Chemother* 1989;23:817–23.